Triclocarban (TCC), CAS no. 101-20-2

Synonyms: Tcc

Substance type: TCC (Figure 1); 3,4,40-trichlorocarbanilide; 3-(4-Chlorophenyl)-1-(3,4-dichlorophenyl)urea).
TCC is an antimicrobial agent used widely in various personal hygiene products including soaps, toothpaste and shampoo and is used in 100-1000 tonnes per annum. TCC is remarkably stable and resistant to biological and chemical treatments, so there is also a potential for exposure by ingestion of contaminated water and/or agricultural products exposed to TCC-containing sludge (Duleba et al 2011). Several studies have reported that serum concentrations of triclocarban in humans are in the nM range (from Wu et al. 2016).

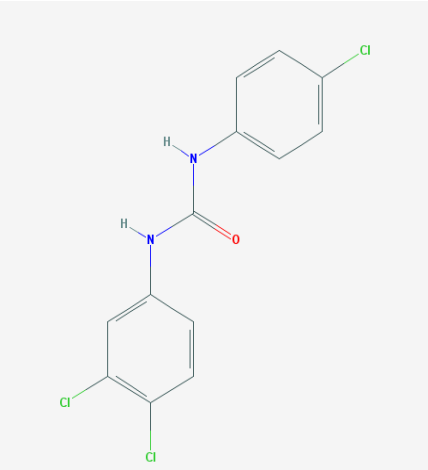

Figure 1. 2D structure from PubChem

4. Human health hazard assessment

4.10.3 Endocrine disruption

4.10.3.1 General approach – human health

4.10.3.2 In vitro information indicative of endocrine activity

Wu et al. (2016)
This study examined the effects of TCC on sodium/iodide symporter (NIS)-mediated iodide uptake and the expression of genes involved in thyroid hormone (TH) synthesis in rat thyroid follicular FRTL-5 cells (at concentrations from 0.01 to 1000 µM), and on the activity of thyroid peroxidase (TPO) using rat thyroid microsomes. TCC inhibited NIS-mediated iodide uptake in a concentration-dependent manner at ranges below effects on cell viability. A decrease in the iodide uptake was also observed in the absence of sodium iodide (NaI) at non-cytotoxic concentrations. Kinetic studies showed that TCC is a non-competitive inhibitor of NIS. FRTL-5 cells were also used to measure the transcriptional expression of three genes involved in TH synthesis, Slc5a5, Tpo, and Tgo, as well as three thyroid transcription factor genes, Pax8, Foxe1, and Nkx2-1. No significant changes in the expression of any genes were observed for TCC. Furthermore, TCC inhibited the activity of TPO in a concentration-dependent manner, however with a low potency, i.e. IC50 of >300 µM. The observations that TCC inhibited NIS-mediated iodide uptake, but did not influence the transcriptional expression levels of Slc5a5, the mRNA that codes for NIS, suggest that TCC may affect NIS at the post-translational level. When FRTL-5 cells were incubated with TCC in the absence of NaI, inhibition of iodide uptake was observed at as low as 0.3 µM. This concentration was much lower than the IC50 value for iodide uptake (17.2 µM) when FRTL-5 cells were exposed to the TCC and NaI simultaneously, and this indicates that inhibition of iodide uptake by TCC is mediated primarily through regulating NIS at the post-translational level. Although TCC inhibited the TPO activity in a concentration-dependent manner, TPO seemed not a primary target of TCC, because the IC50 value for TPO activity inhibition (>300 µM) was higher than those for iodide uptake. In summary, TCC acted as non-competitive inhibitors of NIS and inhibited NIS-mediated iodide uptake in FRTL-5 cells. Compared to iodide uptake, TPO seemed not to be a primary target of TCC.

Study quality and assessment: TCC inhibited NIS-mediated iodide uptake in a concentration-dependent manner has been shown at ranges below effects on cell viability. Kinetic studies showed that TCC is a non-competitive inhibitor of NIS. TCC inhibited the TPO activity in a concentration-dependent manner, but compared to iodide uptake, TPO seemed not to be a primary target of TCC. Overall, the quality of the study is evaluated as high and the in vitro evidence for NIS inhibition is evaluated as strong.

Kolšek et al. (2015)
The effect of TCC on glucocorticoid receptor (GR) antagonistic activity was investigated using the MDA-kb2 cell line stably transfected with MMTV luciferase reporter gene. TCC at concentrations of 2µM was shown to enhance hydrocortisone (HC) (500nM) induced luciferase activity to ~156% compared to control (DMSO 100%). The same effect was seen when investigating TCC androgen receptor (AR) antagonistic activity. 2 µM TCC enhanced dihydrotestosterone (DHT) induced signal to ~166% when co-administrated with 0.5 nM DHT.

Study quality and assessment: The study is well-written although some details on methods are described in the results section. The chosen assays appear to be well performed, e.g. the tested concentrations of TCC were below cytotoxicity and each experiment was at least performed in duplicates. However, a study has shown that TCC stabilizes the luciferase enzyme, which may have confounded the read-outs from the AR reporter gene assay. The study is thus assessed to be of medium quality. This study provides weak evidence that TCC acts as a modulator of the GR and AR activity.

Huang et al. (2014)
TCC exerted estrogenic activity by inducing luciferase activity in an ER reporter gene assay, promoting the proliferation of human mammary carcinoma MCF-7 cells, up-regulating the expression of estrogen-inducible pS2 gene and down-regulating ERα expression at both mRNA and protein levels in the MCF-7 cells. Furthermore, TCC could alter the expression of multiple microRNAs (mir-22, mir-206 and mir-193b) in the MCF-7 cells.

Study quality and assessment: The study is well-described, all assays were performed in triplicates and included both positive and vehicle controls. However, more information on CAS-number would have been preferred. Cytotoxicity was not evaluated but most of the results including increased proliferation, increased luciferase activity, increased protein levels (of pS2) and upregulation of gene expression (of pS2) and microRNA expression levels indicate that cytotoxicity is not present at the concentration tested. Similar TCC concentrations were used in all assays and cytotoxicity is not considered an issue in this study. A study has shown that TCC stabilizes the luciferase enzyme which may have confounded the read-out on luciferase activity and the results from the ERα reporter gene assay has limited scientific value. The study is assessed to be of medium quality. The study provides moderate evidence of an estrogenic mode of action *in vitro*.

Tarnow et al. (2013)

In a MDA-kb2 reporter cell line transfected with a stable MMTV.luciferase.neo reporter gene construct, 1µM[1] TCC was shown to induce androgen-mediated amplification of luciferase-activity by ~40%. 1µM of TCC was also shown to induce estrogen-mediated amplification of luciferase-activity by ~50% in HeLa9903 cells stably transfected with human estrogen receptor (ER)α and an estrogen responsive element (ERE) driven luciferase reporter gene. TCC did not affect the transcription of genes known to be regulated by androgen receptor (AR): specifically androgen-regulated gene protein (SARG), N-myc downstream regulated 1 (NDRG1) and sorbitol dehydrogenase (SORD). Also, no effect of TCC was seen on estrogen responsive genes in human mammary carcinoma MCF-7 cells co-exposed to 17b-estradiol, Bisphenol A, butylparaben or genistein. The induced estrogen mediated enhancement of luciferase-activity was further investigated using an assay (E-screen), where estrogen-dependent cell proliferation is used as the endpoint. These findings show that treatment with E_2 resulted in a dose-dependent increase in MCF-7 cell number, however addition of 1µM TCC was not able to further enhance estrogen-dependent cell proliferation, as was seen with the ~50% enhanced luciferase activity in HeLa9903 cells. Due to the conflicting results, the effect of TCC on firefly luciferase enzyme heat stability was examined using thermal shift. The thermal shift assay showed a stabilizing effect of TCC (above 5µM) on the luciferase enzyme, which was specifically enhanced in the presence of adenosine triphosphate (ATP) as enzymatic co-factor. The authors argue that in cellular assays the effective concentration is likely to be lower due to more physiological buffered conditions. Thus, these results point to the effects of TCC on ER and AR as being false positive due to a stabilizing effect of TCC on the luciferase enzyme. TCC showed a co-stimulatory effect on transcription of CYP1A1 and CYP1B1, which are classical target genes of the transcription factor aryl hydrocarbon receptor (AhR). The authors argue that TCC interfere with AhR, which are connected to ERα via complex regulatory crosstalk, and interference due to AhR-ER crosstalk may lead to adverse outcomes (reviewed in Tarnow et al. 2013).

Study quality and assessment: The study is well described and the authors appear to be thorough in the methods used. Each assay was performed in triplicates, they include positive and negative controls, and they verified the presence and absence of relevant receptors (e.g. AR and ER) in the cell lines. As discussed above, TCC stabilizes the luciferase enzyme and this may have confounded the read-out on luciferase activity and the results from the AR reporter assay and the ER reporter gene assay have limited scientific value. Moreover, they did not test for cytotoxicity. Finally, more information on CAS number and purity of the substances would have been preferred. The study is assessed to be of medium quality. The study provides weak evidence of an endocrine disrupting mode of action of TCC.

Duleba et al. (2011)

Summary: Luciferase reporter plasmids containing probasin (probasin-luc) or three repeats of the androgen response element ligated to the luciferase reporter (ARE-luc) was transfected into LNCaP and C4-2B cells (human prostate cancer cell lines). The transfected cells were exposed to testosterone (T) (1nmol/L), dihydrotestosterone (DHT) (1nmol/L), TCC (1µmol/L) or a combinational treatment of TCC + T or TCC + DHT. Furthermore, the signal transduction independency via androgen receptor (AR) was tested using the AR inhibitor, biclutamide. T and DHT significantly increased luciferase activity in both LNCaP and C4-2B cells containing ARE-luc or probasin-luc. TCC alone had no effect on luciferase activity, however in combination with androgens, the luciferase activity was further

[1] corresponds to the maximal levels realistically expected in human blood – stated by the authors

increased by 221% (probasin promotor, DHT) and 175% (ARE promoter, T) in LNCaP cells compared to androgen treatment alone. The same enhancement of luciferase activity was seen in C4-2B cells where TCC + androgen co-treatment further enhanced luciferase activity by 25.9% (probasin promotor, DHT) and 38.5% (ARE promoter, T) compared to androgen treatment alone. Furthermore, the AR binding inhibitor, bicalutamide, significantly suppressed the enhanced effect of TCC and androgens down to levels comparable to when TCC was administrated alone.

Study quality and assessment: The study is well-described and is assessed to be of high quality. However, a study has shown that TCC stabilizes the luciferase enzyme which may have confounded the effect. This study provides moderate evidence for amplification of androgen activity.

Hinther et al. (2011)

Summary: This study assessed the effects of TCC (10, 100 or 1000 nM), alone or in combination with triiodothyronine (T3) (10 nM), on thyroid hormone (TH) signaling and cellular stress using the cultured frog tadpole tail fin biopsy (C-fin) assay and the TH-responsive rat pituitary GH3 cell line. mRNA abundance of TH receptor β (TRβ), Rana larval keratin type I (RLKI), both indicating a TH response was measured in the C-fin assay. The TH-responsive gene transcripts encoding growth hormone (Gh), deiodinase I (Dio1), and prolactin (Prl) were measured in the GH3 cells. In the C-fin assay, TCC alone significantly decreased RLKI transcript levels ($p = 0.021$) at the highest concentration (1000 nM) but did not affects TRβ transcript levels. In the presence of T3, TCC did not affect TRβ or RLKI steady-state transcript levels. In the GH3 cells, TCC (1000 nM), alone or in the presence of T3, significantly reduced the levels of TH-responsive gene transcripts: Gh, Dio1 and Plr.

Study quality and assessment: This study has been criticized by Fort et al. (2011) and DeLeo et al. (2011) for inadequate/wrong citations and the applied method, respectively. DeLeo et al. (2011) further noted that since TCC induced down-regulation of RLKI, but did not alter TRβ, TCC should not necessarily be considered a disruptor of thyroid axis without further evidence. Overall, the quality of the study is evaluated as low and the evidence of TH MoA is evaluated as weak.

Christen et al. (2010)

Summary: MDA-kb2 cells were stably transfected with murine mammalian tumor virus (MMTV)-luciferase. TCC was analyzed in concentration that was previously shown not to be cytotoxic. In the analysis of anti-androgenic activity the MDA-kb2 cells were co-exposed to dihydrotestosterone (DHT) and TCC (+ a solvent control (negative control) and DHT (positive control)). Luciferase activity was measured after 24 h of incubation. TCC showed cytotoxicity at concentrations higher than 5 µM. TCC showed no androgenic activity in itself, but potentiated the DHT response (0.5nM) up to 130% at 0.01-5 µM TCC. Addition of 10µM flutamide inhibited the induction of this response, thereby excluding that the potentiation is due to/originates from co-activation of the glucocorticoid receptor (GR) that can also stimulate the expression of luciferase by TCC.

Study quality and assessment: The study is well-described and is assessed to be of high quality. However, a study has shown that TCC stabilizes the luciferase enzyme which may have confounded the effect. This study provides moderate evidence for amplification of androgen activity.

Chen et al. (2008)
Summary: The androgen and/or anti-androgenic activity of TCC was investigated using human embryonic kidney 293 (2933Y) cells, which were transfected with PCDNA6-human androgen receptor (AR) and MMTV-Luc.neo plasmid containing a luciferase reporting gene. No cell cytotoxicity was detected for TCC when administrated alone or in combination with 0.125 nM testosterone (T). When administered alone TCC (1.0 µM) showed no effect on transcriptional luciferase activity, however in combination with T (0.125nM), TCC amplified the T-induced transcriptional activity by 45%. This amplifying effect was both time and dose dependent. Flutamide, a competitive inhibitor of androgen binding to the AR, suppressed this amplification effect of 1.0µM TCC at a flutamide concentration of 10uM.
TCC was tested in a competitive binding assay at multiple concentrations up to 200 µM, but showed no competitive binding to the AR. Also, TCC did not activate cyclic adenosine monophosphate (cAMP)/ protein kinase A (PKA)-mediated luciferase activity or enhanced the signal transduction induced by human chorionic gonadotropin (hCG).
The effect of TCC on AR protein expression was investigated by western blot analysis. MDA-kb2 and 2933Y cells were treated for 48h with either 0.1 nM T, 1.0 µM TCC or a combination of T (0.1 nM) and TCC (1.0 µM). Subsequently, western blot analysis was conducted on cell lysates. Results show that treatment with T as well as T+TCC increased AR immunoreactive (AR-ir) protein in both cell lines compared to vehicle control. In the MDA-kb2 cells TCC + T resulted in more AR-ir protein compared to treatment with T alone. However, in 2933Y cells no statistical difference on AR-ir protein was observed between the T and T+TCC treatment. The authors state that this difference could be due to the inherent differences between the exogenous AR in the 2933Y cells and the endogenous AR MDA-kb2 cells.

Study quality and assessment: The study is well-described and is assessed to be of high quality. However, a study has shown that TCC stabilizes the luciferase enzyme which may have confounded the results. This study provides moderate evidence for amplification of androgen activity in two different in vitro assays.

Ahn et al. (2008)
Summary: 1 µM TCC was shown to significantly enhance estradiol (E_2) induced luciferase activity in recombinant human ovarian cells [BG1Luc4E_2, ER-α-positive] containing estrogen receptor (ER)-responsive firefly luciferase reporter gene plasmid, pGudLuc7ERE, when E_2 was co-administrated at concentrations of 1-10 nM. Furthermore, TCC (1µM) enhanced testosterone dependent induction of AR-mediated luciferase gene activity in recombinant human cells [T47D-androg-responsive-element (ARE)] containing AR-responsive firefly luciferase reporter gene plasmid, pGudLuc7ARE, but only at testosterone concentrations of 10µM (the highest concentrations). TCC showed weak ER activity at concentrations of 1-10µM.

Study quality and assessment: The study is well-described and is assessed to be of high quality. However, a study has shown that TCC stabilizes the luciferase enzyme which may have confounded the effect. This study provides moderate evidence for weak ER activity.

4.10.3.3 In vivo effects with regard to an endocrine mode of action

Kennedy et al. (2015)

Summary: This study (from the same group as Chen et al. 2008 and Duleba et al. 2011) investigated how exposure to TCC during early life affects the trajectory of fetal and/or neonatal survival and development. Sprague Dawley rats were provided control, 0.2% weight/weight (w/w), or 0.5% w/w TCC-supplemented chow through a series of 3 experiments that limited TCC exposure to critical growth periods: 1) gestation, 2) gestation and lactation, or 3) lactation only (cross-fostering). In the first experiment, the level of TCC was measured in serum and amniotic fluid of gestation day (GD) 19 in dams fed from GD5-19 with either TCC-supplemented (n=5 in each group) or standard chow (n=4). They also assessed circulating hormone levels (E_2, progesterone, T, triiodothyronine (T3), thyroxine (T4) and thyroid-stimulating hormone (TSH)), number of implantation sites, systemic (liver, kidney and adrenal) organ weights, and sex organ (ovary) weights and histology. The only observed effect from TCC exposure was a decreased T3 level in the 0.5% TCC group. In the second experiment, the effect of *in utero* and lactational exposure to TCC on neonate survival was first examined by feeding dams either standard or TCC-supplemented chow from GD5 until weaning at postnatal day (PND) 21 (n=5 in each group). While pups born and raised by control rats survived beyond weaning, pups born and raised by TCC-treated dams did not survive beyond PND8. Histological examinations of mammary glands collected from the TCC-treated dams showed evidence of increased lobule separation by interstitial mature fat, increase in epithelial vacuolation with fat and thinning of epithelial height. To further investigate if the effect of TCC exposure on mammary tissue and reduced lactation was involved in the reduced neonatal survival a new study was performed. Here daily estimates of the size of milk band on pups were used as an indicator for the amount of milk consumed by pups. After PND3 a decrease in milk band size was observed in the pups born/raised by dams receiving 0.5%TCC compared to control pups born/raised by control dams. The concentration level of TCC in dam milk and serum from dams and pups was measured during lactation. Interestingly, the level of TCC in the milk was almost four times higher than serum levels of TCC in the dams. Finally, in the third experiment, the effect of TCC exposure during lactation on the survival of F1 female pups was investigated in a cross-fostering study. The results from this study show decreased survival and body weight as well as distended abdomen and diarrhea of pups raised by TCC-treated dams compared to pups raised by control dams, regardless of their *in utero* exposure. No effect of TCC was seen on anogenital distance (AGD), vaginal opening (VO) date, or first date of estrus after VO in any of the exposure scenarios.

Study quality and assessment: The quality of the study is evaluated as low due to the low number of litters per group (N=3-5). Thus, the lack of effect of TCC on anogenital distance, vaginal opening (VO) date, or first date of estrus after VO as well as the observed decrease of T3 level do not provide evidence for absence or presence of adverse endocrine effects of TCC.

Duleba et al. (2011)

Summary: This study was an extension of the study by Chen et al. 2008. In this study the same group of researchers examined whether TCC induces effects on intact peripubertal male rats. Twenty-four intact male SD rats aged 48-52 days were randomly assigned into two groups with 12 rats in each group: a control group receiving normal standard diet, and a TCC group receiving 0.25% (2500 ppm, 2500 mg/kg diet) TCC in the diet for 10 days. Rats receiving TCC in the diet had a significantly higher absolute and relative weight of all accessory sex organs except from the testes compared to

control rats. The relative weight of the seminal vesicle was increased by ~38%, ventral prostate by ~25%. Levator ani-bulbocavernosus (LABC) muscle by ~126% and glands penis by ~29%. The absolute and relative liver weight was also significantly increased by ~14% and ~8%, respectively, and post-treatment body weight (BW) was non-significantly increased by ~5% when compared to the control group. This increased organ weight seen in TCC treated animals was also applicable for the seminal vesicle, LABC muscle and glands penis when looking at dry weight. The kidney also had a small but significantly increase in dry weight even though this significant increase was not seen in the other weight measures. Furthermore, the TCC group also had significantly higher protein and DNA content (mg/organ) of the ventral prostate, LABC muscle and glands penis compared to the control group. When looking at water content of the organs only the LABC muscle had a small but significantly increase in % water. No effects on serum T and luteinizing hormone (LH) levels were measured, and no visible abnormalities or histologically differences of accessory sex organs were observed between treated and control rats.

This paper is included in the REACH registration dossier for Triclocarban

Study quality and assessment: The study is well-described and used a sufficient number of males per group. The study included only two dose groups, which is a limitation with regards to assessment of dose-response relationship. The study is therefore evaluated to be of medium quality.
The study provides strong evidence for endocrine activity, i.e. androgen activity, in intact peripubertal male rats. The marked effect on male reproductive organ weights, in the absence of significant effects on body weight, is suggestive of either cell hypertrophy or hyperplasia. Ventral prostate, LABC and glans penis had significantly increased protein and DNA content indicating that exposure to TCC resulted in increased number of cells per organ, i.e. hyperplasia. The lack of histological differences between accessory sex glands from treated and control rats suggest that the increased growth associated with TCC was proportional in the epithelium and surrounding parenchyma of each organ. Overall, this study is evaluated to provide moderate evidence for adverse reproductive toxicity effects of TCC.

Chen et al. (2008)
Summary: The potential amplification effect of TCC on androgen ligands was investigated *in vivo* using an established and widely used rat model (corresponding to the Hershberger assay) to investigate androgenic/anti-androgenic effects of substances on accessory sex tissue. Castrated 48-52 days old male Sprague Dawley (SD) rats (n=12) were treated for 10 days with sc. injections of testosterone propionate (TP) (0.2 mg/kg bw), TCC (0.25% in the diet, corresponding to 2500 ppm or 2500 mg/kg in the diet), and a combination of TP injections and TCC-supplemented diet. The control group received vehicle (sesame oil) and normal diet. There were no effects of the treatments on body weights. TP treatment alone significantly increased the weight of all accessory sex organs, except from glans penis, compared with the control group and with the group receiving TCC alone. Rats receiving only TCC showed no effect on weight of the seminal vesicles, Cowper's gland, Levator ani-bulbocavernosus (LABC) muscle and glans penis, however a slightly but significantly increased liver weight (~17%) was observed, as well as an increased ventral prostate weight (~47%), when compared to the control group. The co-treatment of TCC and TP showed an additional increase in the weight of all accessory sex organs (~78% for the seminal vesicles, ~67% for the ventral prostate, ~35% for the gland penis, ~65% for the Cowper's gland and ~13% for the LABC muscle) when compared to the effect of TP treatment alone.

Study quality and assessment: The study is well-described, used an established rat model (corresponding to the Hershberger assay) and is evaluated to be of high quality. This study provides strong evidence for endocrine disruptive activity, i.e. amplification of androgen activity, in castrated rats.

REACH registration dossier

Summary: Two potentially relevant studies are mentioned. In the first study, Sprague-Dawley rats (10 rats/sex/group) were dosed with 25% aqueous solution of TCC at 0, 500 or 1000 mg/kg bw by intubation 5 days per week for a thirty day period. Food consumption and weight gain were recorded weekly and observations were made for outward symptoms of toxicity such as reduced activity and non-grooming. At the end of the 30 day period, representative animals from each group were sacrificed. The viscera of the 1000 mg/kg bw and control groups were examined microscopically and saved for possible future examination. Macroscopic examination was made of mounted tissue from liver, kidneys, gonads, adrenals, brain, heart, and lungs. No effects of 1000 mg/kg bw was found on food consumption, growth data, and at the macroscopic tissue examination.

Study quality and assessment: The quality of the study is evaluated as high with regards to the number of doses and animals per dose group. However, the endpoints included are not likely to be sensitive to endocrine disruption and especially the performance of only macroscopic examination of the gonads is of very limited value. Overall, the quality of the study is evaluated as low due to the limited endpoints included. The study does not provide evidence for absence or presence of adverse endocrine effects of TCC.

The second study included in the robust study summary refers Duleba et al. 2011 (see above under the heading Duleba et al 2011).

4.10.3.4 Summary of the plausible link between adverse effects and endocrine mode of action

A vast amount of *in vitro* studies indicate that when administered alone TCC show no estrogenic (Huang et al. 2014, Ahn et al. 2008, Tarnow et al. 2015) or androgenic (Christen et al.2010, Duleba et al. 2011, Ahn et al. 2008, Tarnow et al. 2015, Chen et al. 2008) activity in itself, when using luciferase activity as the indirect read out. When co-administered together with androgens (T or DHT) or estrogens (E_2), TCC induced both androgen- and estrogen-mediated amplification of luciferase activity (Christen et al. 2010, Duleba et al. 2011, Ahn et al. 2008, Tarnow et al. 2015, Chen et al. 2008). The androgen receptor (AR) inhibitors, flutamide and bicalutamide, significantly suppressed the enhanced effect of co-administered TCC and androgens to the levels comparable to control or when TCC was administrated alone (Chen et al. 2008, Christen et al. 2010, Duleba et al. 2011). The amplifying effect of TCC is supported by *in vitro* results on AR protein expression, where co-exposure to TCC + T resulted in a higher expression of AR-ir protein compared to treatment with T alone (Chen et al. 2008). The TCC amplified estrogen-mediated activity from the luciferase activity assay could not be reproduced using the E-screen assay. In contrast another study found that TCC has estrogenic properties in concentrations ranging from $1x10^{-9}$-$1x10^{-6}$M, and that co-treatment with an ER antagonist inhibited the effect of TCC. Furthermore, mRNA analysis using qPCR showed that two estrogen-responsive genes were upregulated (pS2) and downregulated (ERα), respectively, when exposed to TCC, which was confirmed when measuring the protein levels of pS2 and ERα. Studies on micro-RNAs (mir-22, mir-206 and mir-193b), which have recently been identified as potent regulators of ERα in the MCF-7 cells, showed that TCC (and E_2) upregulates the expression of these. A study has shown that TCC stabilizes the luciferase enzyme. This stabilization may explain why estrogenic effects of TCC where seen in the luciferase assay, whereas no effect of TCC was observed in the E-screen or in levels of estrogenic responsive genes. The same confounding effect may explain why TCC with androgens amplified AR activity in the luciferase assay, and is supported by results showing that TCC does not affect transcription of AR regulated genes. TCC showed co-stimulatory effect on transcription of CYP1A1 and CYP1B1, which are classical target genes of the regulon[2] of the aryl hydrocarbon receptor (AhR). This may indicate that TCC interferes with AhR and thereby indirectly affects ERα activity due to AhR-ER crosstalk.

In conclusion, TCC show no estrogenic or androgenic activity in itself in the above mentioned *in vitro* studies (table 1). When co-administered together with androgens (T or DHT) or estrogens (E2), TCC have induced amplification of both androgen- and estrogen-mediated activity in many studies. However, a confounding effect due to TCC-induced stabilization of the luciferase enzyme may partly explain the activity in luciferase assay. Thus, the *in vitro* evidence for estrogenic and androgenic MoA of TCC is evaluated as moderate.

In vivo studies have shown effects of TCC corresponding to the enhancing effects of androgen and estrogen induced activity seen in vitro (Duleba et al. 2011, Chen et al. 2008) (table 2). Investigations in castrated males and intact peripubertal rats show that in presence of androgens, either exogenous or endogenous, TCC further increases the weight of all accessory sex organs compared to the effect of androgen treatment alone (Chen et al. 2008) or control rats (Duleba et al. 2011). Thus, there is both data showing in vivo androgenic MoA of TCC as well as effects typically induced by androgens.

[2] A regulon is a group of genes that are regulated as a unit, generally controlled by the same regulatory gene that expresses a protein acting as a repressor or activator.

The *in vivo* evidence for endocrine MoA is evaluated as strong and moderate evidence for adverse reproductive toxicity effects of TCC.

There is a strong biologically plausible link between the adverse effects in peripubertal male rats and the enhancing effects of androgen and estrogen induced activity seen *in vitro* and especially the androgen MoA seen *in vivo*.

There is *in vitro* evidence for thyroid disturbing MoA of TCC, especially inhibition of NIS. Only one *in vivo* study of low quality has investigated relevant endpoints for thyroid effects and found decreased triiodothyronine (T3) in the absence of effects on thyroxine (T4) or TSH. This does not provide *in vivo* evidence for thyroid effects of TCC. As the *in vivo* evidence is evaluated as absent/weak, TCC is not evaluated to fulfil the WHO-definition for being considered as an ED with a thyroid MoA. However, TCC is evaluated as suspected ED with a thyroid MoA based on the *in vitro* data.

There are two studies on MoA related to effects on the thyroid axis. Indications of thyroid disruption have been reported by Hinther *et al.*, 2011, but the study is evaluated to have low quality and the results appear unclear, i.e. provide only weak evidence. TCC inhibited NIS-mediated iodide uptake in a concentration-dependent manner has been shown at ranges below effects on cell viability (Wu et al. 2016). The, the *in vitro* evidence for TH MoA manifested as NIS inhibition is considered strong.

In conclusion, triclocarban meets the WHO-definition of an endocrine disruptor with an androgenic MoA.

Table 1. Overview of *in vitro* and *in vivo* endocrine disrupting (ED) mode(s) of action (MoA(s)) of triclocarban

Reference	**MoA**		**Quality of study**	**Evidence for ED MoA**
	In vitro	*In vivo*		
Wu et al. 2016	TCC acted as non-competitive inhibitors of NIS and inhibited NIS-mediated iodide uptake in FRTL-5 cells. Compared to iodide uptake, TPO seemed not to be a primary target of TCC.		High	Strong
Kolšek et al 2015	TCC was shown to enhance hydrocortisone (HC) (500nM) induced luciferase activity to ~156% compared to control (DMSO 100%). The same effect was seen when investigating TCC androgen receptor (AR) antagonistic activity. 2 µM TCC enhanced dihydrotestosterone (DHT) induced signal to ~166% when co-administrated with 0.5 nM DHT.		Medium	Weak
Huang et al.2014	TCC exerted estrogenic activity by inducing luciferase activity in an ER reporter gene assay, promoting the proliferation of human mammary carcinoma MCF-7 cells, up-regulating the expression of estrogen-inducible pS2 gene and down-regulating ERα expression at both mRNA and protein levels in the MCF-7 cells		Medium	Moderate
Tarnow et al. 2013	TCC was shown to induce androgen-mediated amplification of luciferase-activity by ~40%. no effect of TCC was seen on estrogen responsive genes in human mammary carcinoma MCF-7 cells co-exposed to 17b-estradiol, Bisphenol A, butylparaben or genistein. The induced estrogen mediated enhancement of luciferase-activity was further investigated using an assay (E-screen), where estrogen-dependent		Medium	Weak

Reference	MoA		Quality of study	Evidence for ED MoA
	In vitro	*In vivo*		
	cell proliferation is used as the endpoint. These findings show that treatment with E_2 resulted in a dose-dependent increase in MCF-7 cell number, however addition of 1µM TCC was not able to further enhance estrogen-dependent cell proliferation.			
Duleba et al.2011	TCC alone had no effect on luciferase activity, however in combination with androgens, the luciferase activity was further increased by 221% (probasin promotor, DHT) and 175% (ARE promoter, T) in LNCaP cells compared to androgen treatment alone. The same enhancement of luciferase activity was seen in C4-2B cells where TCC + androgen co-treatment further enhanced luciferase activity by 25.9% (probasin promotor, DHT) and 38.5% (ARE promoter, T) compared to androgen treatment alone. Furthermore, the AR binding inhibitor, bicalutamide, significantly suppressed the enhanced effect of TCC and androgens down to levels comparable to when TCC was administrated alone.		High	Moderate
Hinther et al.2011	In the C-fin assay, TCC alone significantly decreased RLKI transcript levels ($p = 0.021$) at the highest concentration (1000 nM) but did not affects TRβ transcript levels. In the presence of T3, TCC did not affect TRβ or RLKI steady-state transcript levels. In the GH3 cells, TCC (1000 nM), alone or in the presence of T3, significantly reduced the levels of TH-responsive gene transcripts: Gh, Dio1 and Plr.		Low	Weak

Reference	MoA		Quality of study	Evidence for ED MoA
	In vitro	*In vivo*		
Christen et al. 2010	TCC showed no androgenic activity in itself, but potentiated the DHT response (0.5nM) up to 130% at 0.01-5 µM TCC. Addition of 10µM flutamide inhibited the induction of this response, thereby excluding that the potentiation is due to/originates from co-activation of the glucocorticoid receptor (GR) that can also stimulate the expression of luciferase by TCC.		High	Moderate
Chen et al. 2008	Treatment with T as well as T+TCC increased AR immunoreactive (AR-ir) protein in both cell lines compared to vehicle control. In the MDA-kb2 cells TCC + T resulted in more AR-ir protein compared to treatment with T alone. However, in 2933Y cells no statistical difference on AR-ir protein was observed between the T and T+TCC treatment. The authors state that this difference could be due to the inherent differences between the exogenous AR in the 2933Y cells and the endogenous AR MDA-kb2 cells.		High	Strong
Ahn et al. 2008	TCC showed weak ER activity at concentrations of 1-10µM.		High	Moderate

Thyroid peroxidase (TPO), dihydrotestosterone (DHT), androgen receptor (AR), Rana larval keratin type I (RLKI), TH receptor β (TRβ), triiodothyronine (T3), testosterone (T), estrogen receptor (ER)

Table 2. Overview of potential endocrine-related adverse effects of triclocarban.

Reference	Species, n	Adverse effects	Quality of study	Evidence for adverse effects
Kennedy et al. 2015	Rats n = 3-5	The lack of effect of TCC on anogenital distance, vaginal opening (VO) date, or first date of estrus after VO as well as the observed decrease of T3 level do not provide evidence for absence or presence of adverse endocrine effects of TCC.	Low	None
Duleba et al.2011	Rats N=12/group	The marked effect on male reproductive organ weights, in the absence of significant effects on body weight, is suggestive of either cell hypertrophy or hyperplasia. Ventral prostate, LABC and glans penis had significantly increased protein and DNA content indicating that exposure to TCC resulted in increased number of cells per organ, i.e. hyperplasia. The lack of histological differences between accessory sex glands from treated and control rats suggest that the increased growth associated with TCC was proportional in the epithelium and surrounding parenchyma of each organ.	Medium	Moderate

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